



PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN THE APPLICATION OF:

PATRICIA C. BRZOSTOWICZ ET. AL.

CASE CL1903USNA
NO.:

APPLICATION NO.: 09/941947

GROUP ART UNIT: 1645

FILED: AUGUST 29, 2001

EXAMINER: NASHED

FOR: CAROTENOID PRODUCTION FROM A SINGLE CARBON SUBSTRATE

DECLARATION UNDER RULE 132

I, James Martin Odom, do hereby declare that:

1. I obtained my education at University of Georgia College where I received a B.S. in Biochemistry in 1974 and at the University of Georgia, Department of Biochemistry where I received a Ph.D. in Biochemistry in 1981.
2. I have been working at E. I. du Pont de Nemours & Company since 1985 in the area of Biological Sciences. I am currently a Principal Investigator with the Central Research and Development Department.
3. I am familiar with contents of the above named application.
4. As relating to the follow experimental details all assays and analysis were done either by me or under my direction at the E.I. DuPont Experimental Station, Wilmington DE 19898.
5. Below I present a demonstration of (i) a demonstration that methylomonas 16 is a methylotroph, and more specifically a methanotroph; and (ii) expression of the present carotenoid genes is possible in a divergent organism (*E. coli*), suggesting that expression of the present genes in more

related Type I methanotrophs would be within the expectations of the person of skill in the art.

6. The following presents data indicating that *Methylomonas* 16a is classified as a methylotroph, and more narrowly as a methanotroph (capable of using methane as a sole carbon source):

Methylotrophic bacteria (those capable of growing on reduced one-carbon compounds) can be divided into functional groups depending on whether or not they can utilize methane (the subclass called methanotrophs) and on which carbon assimilation pathway is used. The carbon assimilation pathways include the serine pathway and the ribulose monophosphate pathway (RuMP). Both carbon assimilation pathways result in the production of glyceraldehyde-3-phosphate and pyruvate, substrates used in the production of isopentenyl pyrophosphate (see Figure 1 of the present application). The recombinant methylotrophic bacterial host cell exemplified in the working example of the application uses the ribulose monophosphate pathway and also has the ability to use methane, in addition to methanol, as a sole carbon source (Figure 2 of the present application).

Methanotrophs are classified into three metabolic groups ("Type I", "Type X" or "Type II") based on the mode of carbon incorporation, morphology, %GC content and the presence or absence of key specific enzymes. *Methylomonas* 16a is classified as a Type I methanotroph. Table 1 below shows key traits determined for *Methylomonas* 16a in relation to the three major groupings of methanotrophs. The strain clearly falls into the Type I grouping based on every trait, with the exception of nitrogen fixation. It is generally well accepted that these organisms do not fix nitrogen. Therefore, *Methylomonas* 16a appears unique in this aspect of nitrogen metabolism.

Table 1 shows the various properties of *Methylomonas* 16a. The criteria listed in Table 1 are those typically used to determine whether the strain is arbitrarily considered Type I, Type II or Type X based on physical and enzymatic properties. This table was developed from both direct enzymatic assay for enzymes as well as genomic data showing the presence of genes and gene pathways. This categorization is functionally based and indicates that the strain utilizes the most energetically efficient pathway for carbon incorporation which is the ribulose monophosphate or "RuMP" pathway. Genomic data clearly shows the presence of key enzymes in the RuMP pathway. Internal membrane structure

are also indicative of a Type I physiology. Unique to the present strain is the finding of nitrogen fixation genes in *Methylobacter* 16a. The strain is shown to grow in the absence of yeast extract or vitamins. Nitrate, ammonium ion or dinitrogen can satisfy the nitrogen requirement for biosynthesis. This functional data is in complete agreement with the 16sRNA homologies as compared with other *Methylobacter* strains. 16sRNA comparisons of the 16a strain with other *Methylobacter* sp. revealed that *Methylobacter* 16a has 96% identity with the 16sRNA of *Methylobacter* sp. (strain:KSP11) [Hanada, S et al., *J. Ferment. Bioeng.* 86, 539-544 (1998)] and with *Methylobacter* sp. (strain LW13), [Costello, A.M. and Lidstrom, M.E. *Appl. Environ. Microbiol.* 65 (11), 5066-5074 (1999)]. Thus *Methylobacter* 16a is correctly classified as a Type I, RuMP utilizing, *Methylobacter* species.

Table 1

| Characteristic | Type I | <i>Methylobacter</i> s 16a | Type X | Type II |
|---|------------|-------------------------------|------------|----------|
| %GC | Incomplete | Incomplete | Incomplete | Complete |
| Ribmp Cycle | Incomplete | Incomplete | Incomplete | Complete |
| RuBP Carboxylase | - | - | + | + |
| Temp. Range | <45 | <42 | <45 | <40 |
| Nitrogenase | - | + | + | + |
| G6P dehydrogenase e NADP | + | + | + | - |
| Isocitrate dehydrogenase e NAD/NADP | + | + | - | - |
| Yeast Extract | - | - | - | - |
| Vitamins | - | - | - | - |
| Pigmentation | Variable | + | Variable | Variable |
| Nitrate assimilation | + | + | + | + |
| | | | | |

Method of enzymatic assay

Nitrogenase was not assayed but is considered positive if the gene is present on the basis of genome sequence analysis.

Glucose 6 phosphate dehydrogenase: One mL of reaction mixture contains 100 μ L of 10 mM NADP, 100 μ L of 10 mM glucose, 700 μ L of 100 mM HEPES pH 7 buffer and up to 100 μ L of enzyme extract. The enzyme activity was measured by monitoring NADP reduction to NADPH at 340 nm using spectrophotometer.

Isocitrate dehydrogenase: One mL of reaction mixture contains 100 μ L of 10 mM sodium isocitrate, 100 μ L of 10 mM NADP, 700 μ L of 100 mM pH 7 HEPES buffer up to 100 μ L of enzyme extract. The enzyme activity was measured by monitoring NADPH formation at 340 nm.

Nitrate assimilation is based on the ability of the strain to grow on nitrate as sole nitrogen source.

7. The following data demonstrate the expression of carotenoid genes in *E. coli* and support the premise that the person of skill in the art, with evidence that these genes are expressed in a methylotrophic bacterium, as well as a divergent bacteria such as *E. coli*, would reasonably expect similar expression in all Type I methanotrophs.

The genes of the lower carotenoid biosynthetic pathway (*crtE*, *X*, *Y*, *I*, *B*, and *Z*) were cloned from *Pantoea stewartii* as described in Example 6 of the specification. Expression of these genes and analysis of gene function by transposon mutagenesis is described below:

Several plasmids carrying transposons, inserted into each coding region of the *crtE*, *crtX*, *crtY*, *crtI*, *crtB*, and *crtZ* genes were chosen for introduction into *E. coli*. These plasmid variants were transformed to *E. coli* MG1655 and grown in 100 mL Luria-Bertani broth in the presence of 100 ug/mL ampicillin. Cultures were grown for 18 h at 26°C, and the cells were harvested by centrifugation. Carotenoids were extracted from the cell pellets using 10 mL of acetone. The acetone was dried under nitrogen and the carotenoids were resuspended in 1 mL of methanol for HPLC analysis. A Beckman System Gold® HPLC with Beckman Gold Nouveau Software (Columbia, MD) was used for the study. The crude extraction (0.1 mL) was loaded onto a 125 x 4 mm RP8 (5 μ m particles) column with corresponding guard column (Hewlett-Packard, San Fernando, CA). The

flow rate was 1 mL/min, while the solvent program used was: 0-11.5 min 40% water/60% methanol; 11.5-20 min 100% methanol; and 20-30 min 40% water/60% methanol. The spectrum data were collected by a Beckman photodiode array detector (model 168).

In the wild-type clone with wild-type *crtEXYIBZ*, the carotenoid was found to have a retention time of 15.8 min and an absorption spectra of 450 nm, 475 nm. This was the same as the β -carotene standard. This suggested that the *crtZ* gene organized in the opposite orientation was not expressed in this construct. The transposon insertion in *crtZ* had no effect as expected (data not shown).

HPLC spectral analysis also revealed that a clone with transposon insertion in *crtX* also produced β -carotene. This is consistent with the proposed function of *crtX* encoding a zeaxanthin glucosyl transferase enzyme at a later step of the carotenoid pathway following synthesis of β -carotene.

The transposon insertion in *crtY* did not produce β -carotene. The carotenoid's elution time (15.2 min) and absorption spectra (443 nm, 469 nm, 500 nm) agree with those of the lycopene standard. Accumulation of lycopene in the *crtY* mutant confirmed the role of *crtY* as a lycopene cyclase encoding gene.

The *crtI* extraction, when monitored at 286 nm, had a peak with retention time of 16.3 min and with absorption spectra of 276 nm, 286 nm, and 297 nm, which agrees with the reported spectrum for phytoene. Detection of phytoene in the *crtI* mutant confirmed the function of the *crtI* gene as one encoding a phytoene dehydrogenase enzyme.

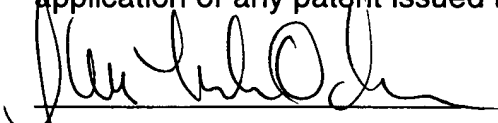
The extraction of the *crtE* mutant, *crtB* mutant and *crtI* mutant was clear. Loss of pigmented carotenoids in these mutants indicated that both the *crtE* gene and *crtB* gene are essential for carotenoid synthesis. No carotenoid was observed in either mutant, which is consistent with the proposed function of *crtB* encoding a prephytoene pyrophosphate synthase and *crtE* encoding a geranylgeranyl pyrophosphate synthetase. Both enzymes are required for β -carotene synthesis.

Results of the transposon mutagenesis experiments are shown below in Table 2. The site of transposon insertion into the gene cluster *crtEXYIB* is recorded, along with the color of the *E. coli* colonies observed on LB plates, the identity of the carotenoid compound (as determined by HPLC spectral analysis), and the experimentally assigned function of each gene.

Table 2

| Transposon insertion site | Colony color | Carotenoid observed by HPLC | Assigned gene function |
|--|--------------|-----------------------------|---|
| Wild Type (with no transposon insertion) | Yellow | β -carotene | |
| <i>crtE</i> | White | None | Geranylgeranyl pyrophosphate synthetase |
| <i>crtB</i> | White | None | Prephytoene pyrophosphate synthase |
| <i>crtI</i> | White | Phytoene | Phytoene dehydrogenase |
| <i>crtY</i> | Pink | Lycopene | Lycopene cyclase |
| <i>crtZ</i> | Yellow | β -carotene | β -carotene hydroxylase |
| <i>crtX</i> | Yellow | β -carotene | Zeaxanthin glucosyl transferase |

8. Further, I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.


(James Martin Odom)

Date 10/11/2004



PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN THE APPLICATION OF:
BRZOSTOWICZ ET AL.

CASE NO.: CL1903 US NA

APPLICATION NO: UNKNOWN

GROUP ART UNIT: UNKNOWN

FILED: CONCURRENTLY HEREWITH

EXAMINER: UNKNOWN

TITLE: CAROTENOID PRODUCTION FROM A SINGLE CARBON SUBSTRATE

Assistant Commissioner for Patents
Washington, D.C. 20231**DECLARATION OF BIOLOGICAL CULTURE DEPOSIT**

Sir:

I, S. Neil Feltham, declare that:

I am an attorney of record for the owner of the above-identified application.

Cultures of the following biological materials have been deposited with the following international depository:

American Type Culture Collection (ATCC)

10801 University Boulevard, Manassas, VA, 20110-2209, U.S.A.

under conditions that satisfy the requirements of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure. A copy of the receipt issued pursuant to Rules 7.3 and 10.2 (stating the term of the deposit) is attached.

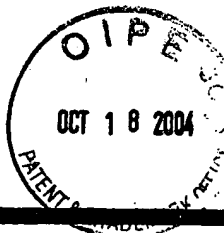
| <u>Depositor's Identification of Organism</u> | <u>International Depository Accession Number</u> | <u>Date of Deposit</u> |
|---|--|------------------------|
| <i>Methylomonas: Methylomonas 16a sp.</i> | PTA-2402 | August 22, 2000 |

I further aver that all restrictions on the availability to the public of the culture will be irrevocably removed upon the granting of a U.S. patent on the above-identified application.

Respectfully submitted,

S. NEIL FELTHAM
ATTORNEY FOR APPLICANTS
US PTO REGISTRATION NO. 36,506
TELEPHONE: (302) 992-6460Dated: 9/25/01
Enclosure: Deposit Receipt

ATCC



10801 University Blvd • Manassas, VA 20110-2209 • Telephone: 703-365-2700 • FAX: 703-365-2745

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF
THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT ISSUED PURSUANT TO RULE 7.3
AND VIABILITY STATEMENT ISSUED PURSUANT TO RULE 10.2

To: (Name and Address of Depositor or Attorney)

DuPont Company
Attn: Kyungok Wun-Kim
DuPont Experimental Station
E301/317
Willmington, DE 19880

Deposited on Behalf of: DuPont Company

Identification Reference by Depositor:
Methylomonas: Methylomonas 16a sp.

Patent Deposit Designation
PTA-2402

The deposit was accompanied by: a scientific description a proposed taxonomic description indicated above.

The deposit was received August 22, 2000 by this International Depository Authority and has been accepted.

AT YOUR REQUEST: X We will inform you of requests for the strain for 30 years.

The strain will be made available if a patent office signatory to the Budapest Treaty certifies one's right to receive, or if a U.S. Patent is issued citing the strain, and ATCC is instructed by the United States Patent & Trademark Office or the depositor to release said strain.


If the culture should die or be destroyed during the effective term of the deposit, it shall be your responsibility to replace it with living culture of the same.

The strain will be maintained for a period of at least 30 years from date of deposit, or five years after the most recent request for a sample, whichever is longer. The United States and many other countries are signatory to the Budapest Treaty.

The viability of the culture cited above was tested September 8, 2000. On that date, the culture was viable.

International Depository Authority: American Type Culture Collection, Manassas, VA 20110-2209 USA.

Signature of person having authority to represent ATCC:



Frank Simione, Director, Patent Depository

Date: November 16, 2000

cc: S. Neil Feltham (Ref: Docket or Case No.: BC-1039)